Volume 295, number 1,2,3, 51–54 FEBS 10502 December 1991

Uncoupling effect of fatty acids on heart muscle mitochondria and submitochondrial particles

V.I. Dedukhova[1], E.N. Mokhova[1], V.P. Skulachev[1], A.A. Starkov[1], E. Arrigoni-Martelli[2] and V.A. Bobyleva[3]

[1]*Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, USSR,* [2]*Sigma-Tau, Pomesia, Roma, Italy* and [3]*Institute of General Pathology, Modena, Italy.*

Received 10 October 1991

The effect of ATP/ADP-antiporter inhibitors on palmitate-induced uncoupling was studied in heart muscle mitochondria and inside-out submitochondrial particles. In both systems palmitate is found to decrease the respiration-generated membrane potential. In mitochondria, this effect is specifically abolished by carboxyatractylate (CAtr) a non-penetrating inhibitor of antiporter. In submitochondrial particles, CAtr does not abolish the palmitate-induced potential decrease. At the same time, bongkrekic acid, a penetrating inhibitor of the antiporter, suppresses the palmitate effect on the potential both in mitochondria and particles. Palmitoyl-CoA which is known to inhibit the antiporter in mitochondria as well as in particles decreases the palmitate uncoupling efficiency in both these systems. These data are in agreement with the hypothesis that the ATP/ADP-antiporter is involved in the action of free fatty acids as natural uncouplers of oxidative phosphorylation.

Uncoupling; Fatty acid; ATP/ADP-antiporter; Heart mitochondria; Submitochondrial particle

1. INTRODUCTION

Fatty acids are known to differ from classical protonophore uncouplers in that they penetrate through a phospholipid bilayer only in their protonated form. Their anions do not penetrate the bilayer [1,2]. This is why they fail to uncouple, i.e. to increase the H^+ conductance, in cytochrome oxidase proteoliposomes [2]. To explain the mechanism of fatty acid uncoupling in mitochondria, we suggested that there are protein(s) in the inner mitochondrial membrane that facilitate transport of the fatty acid anionic species [2–5].

In brown fat, which is specialized for heat production by means of fatty acid-mediated uncoupling, the role of fatty acid anion porter was postulated to be performed by the 'uncoupling protein' thermogenin [5].

Recently this suggestion seems to have been confirmed by Jezek and Garlid [6,7] who found that thermogenin transports various monovalent anions which can substitute for fatty acids in actuating thermogenin-linked H^+-conducting activity. The anion efficiency was shown to increase with the increase in the length of hydrocarbon chain of the anion.

Thermogenin is absent from tissue other than brown fat, but nevertheless fatty acids can uncouple in these other tissues (for reviw, see [5]). We assumed [2,5] that in these cases the role of the fatty acid anion porter is performed by ATP/ADP antiporter, a protein which is very similar to thermogenin in amino acid sequence, domain structure, and some other properties [8,9]. It was suggested that the adenine nucleotide anion-translocating machinery of the antiporter can transport hydrophobic anions without the involvement ATP^{4-} (ADP^{3-})-specific gate [2,5]. In agreement with this hypothesis, the following observations were made by our group. (1) The ATP/ADP antiporter inhibitors (CAtr, Atr, bongkrekic acid and pyridoxal 5-phosphate) and its substrate (ADP anion) specifically inhibit fatty acid-induced uncoupling in liver and skeletal muscle mitochondria, while having no effect on FCCP-induced uncoupling [2–4]. (2) CAtr-sensitive uncoupling is inherent not only to fatty acids but also to other hydrophobic monovalent anions such as dodecyl sulfate and cholate [10]. (3) The burst in heat production during arousal of the hibernating ground squirrels is accompanied by (i) appearance of CAtr-sensitive uncoupling in liver, heart and skeletal muscle mitochondria and (ii) an increase of the free fatty acid concentration in tissues and isolated mitochondria [11–12].

The observation (1) was recently confirmed and extended by Schonfeld [13] who showed that the uncou-

Abbreviations: $\Delta\psi$, transmembrane electric potential difference; Atr, atractylate; BSA, bovine serum albumin; CAtr, carboxyatractylate; DNP, 2,4-dinitrophenol; EDTA, ethylenediamine-*N,N,N'N'*-tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; MOPS, morpholinopropane sulphonate; PCB^-, phenyldicarbaundecaborane; SDS, sodium dodecyl sulfate; TPP^+, tetraphenyl phosphonium

Correspondence address: V.P. Skulachev, Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, USSR

pling activity of a fatty acid varies in different tissues in proportion to the ATP/ADP antiporter content (heart > kidney > liver).

In this paper, we compared the uncoupling effect of fatty acids in heart muscle mitochondria and inside-out submitochondrial particles.

2. MATERIALS AND METHODS

Mitochondria were isolated from rabbit or heart muscle. Chilled muscle tissue, previously separated from fat and tendons, were minced and then passed through a stainless-steel press with holes of about 1 mm in diameter.

The tissue was then homogenized using a Teflon pestle in a glass (Pyrex) homogenizer for about 3 min. The tissue/isolation medium ratio was 1:8.

After the first centrifugation (10 min, 600×g), the supernatant was decanted and filtered through 4 layers of gauze. The filtrate was centrifuged (10 min, 12 000×g), the pellet resuspended in 1 ml of the isolation medium (250 mM sucrose, 10 mM MOPS, 1 mM EDTA, pH 7.4) supplemented with BSA (3 mg/ml), and then medium without BSA was added. The final mitochondrial precipitate (10 min, 12 000×g) was suspended in isolation medium with BSA. The mitochondrial suspension (70–90 mg/ml) was stored on ice.

Inside-out AS-type submitochondrial particles were prepared according to Kotlar and Vinogradov [14].

In the majority of experiments, the incubation medium contained 250 mM sucrose, 10 mM MOPS, and 0.5 mM EGTA, pH 7.4. Glutamate (5 mM) and malate (5 mM) or succinate (5 mM) were used as substrates. 1 μM rotenone was added to the medium when succinate oxidation was studied.

Oxygen consumption was recorded with a Clark-type oxygen electrode and LP-7E polarograph. The incubation mixture was stirred with glass-covered magnetic stirring bar. The concentration of mitochondrial protein was 1 mg/ml (glutamate plus malate) or 0.4 mg/ml (succinate); the temperature was 26°C.

The synthetic penetrating ions (TPP^+ and PCB^-) were used as $\Delta\psi$ probes [15]. The penetrating ion concentration in solution was measured with TPP^+ and PCB^--sensitive electrodes [16]. Oligomycin, MOPS, palmitic acid, palmitoyl-CoA, CAtr, Atr and fatty acid-free BSA were from Sigma; EDTA, EGTA, ADP, GDP, rotenone, palmitoyl-L-carnitine and DNP from Serva; malate from Fluka; FCCP from Boehringer.

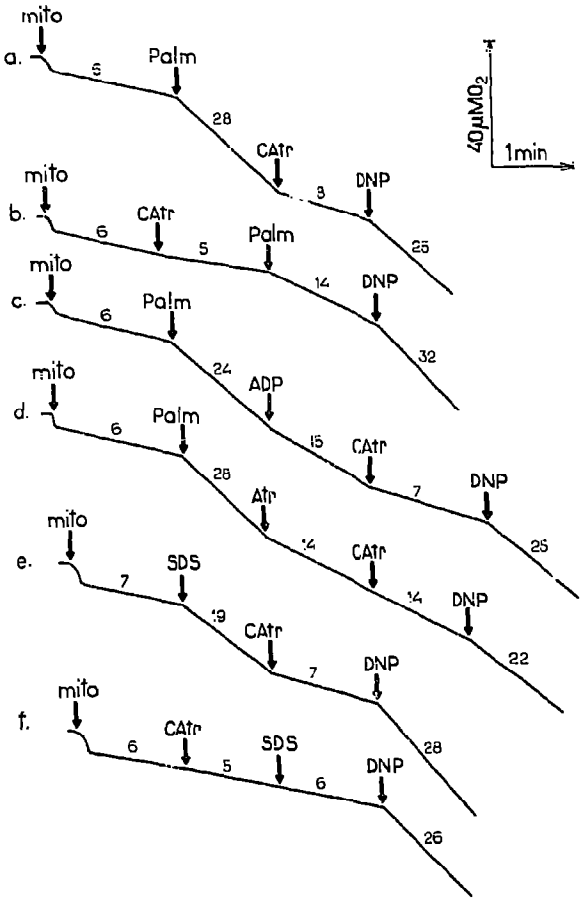

Fig. 1. Effects of CAtr, Atr and SDS on the respiration of heart mitochondria. Incubation mixture: 0.25 M sucrose, 10 mM MOPS, 0.5 mM EGTA, 5 mM glutamate, 5 mM malate, oligomycin (1 μg/ml), BSA (0.2 mg/ml), pH 7.4, 27°C. Additions, mito, a–d, rat heart mitochondria (1 mg protein/ml); e,f, rabbit heart mitochondria (1 mg protein/ml); Palm, 30 μM palmitic acid; 2 μM CAtr; 5 μM Atr; 40 μM ADP; 90 μM SDS; 5 μM DNP (a–d) and 8 μM DNP (e,f). Figures above curves, respiration rates (nmol $O_2 \times min^{-1} \times mg\ protein^{-1}$).

3. RESULTS AND DISCUSSION

As seen in Fig. 1, curves a–c, CAtr was strongly inhibitory for respiration of rat heart mitochondria, stimulated by palmitate in the presence of oligomycin. The inhibition was released by DNP. ADP (curve c), Atr (curve d), palmitoyl CoA and bongkrekic acid (not shown) were also inhibitory but their effects were not as pronounced as that of CAtr. Moreover, CAtr added after Atr failed to inhibit the palmitate-stimulated respiration as strongly as without Atr (curve d). CAtr-sensitive stimulation of respiration could also be induced by SDS but at a concentration higher than that of palmitate (curves e and f). Palmitoyl carnitine could not substitute for either palmitate or CAtr (not shown).

Stimulation of the mitochondrial respiration by palmitate was accompanied by a $\Delta\psi$ decrease which was completely or partially reversed by CAtr or bongkrekic acid, respectively (Fig. 2).

Inside-out beef heart submitochondrial particles were also found to respond to the palmitate addition by a lowering of $\Delta\psi$. However, in this case CAtr did not suppress the action of palmitate. On the other hand, bongkrekic acid suppressed the palmitate effect. Again, as in mitochondria, subsequent addition of an artificial protonophore (FCCP) caused a $\Delta\psi$ decrease (Fig. 3). Palmitoyl CoA proved to decrease the palmitate effect in both mitochondria and submitochondrial particles (not shown).

The data presented above clearly indicate that membrane sidedness is critical for CAtr inhibition of palmitate-induced uncoupling in heart muscle mitochondria and their inside-out particles. CAtr effectively abolishes the uncoupling in the mitochondria but not in the particles. On the other hand, some inhibiting effects of bongkrekic acid and palmitoyl-CoA were seen in both mitochondria and particles.

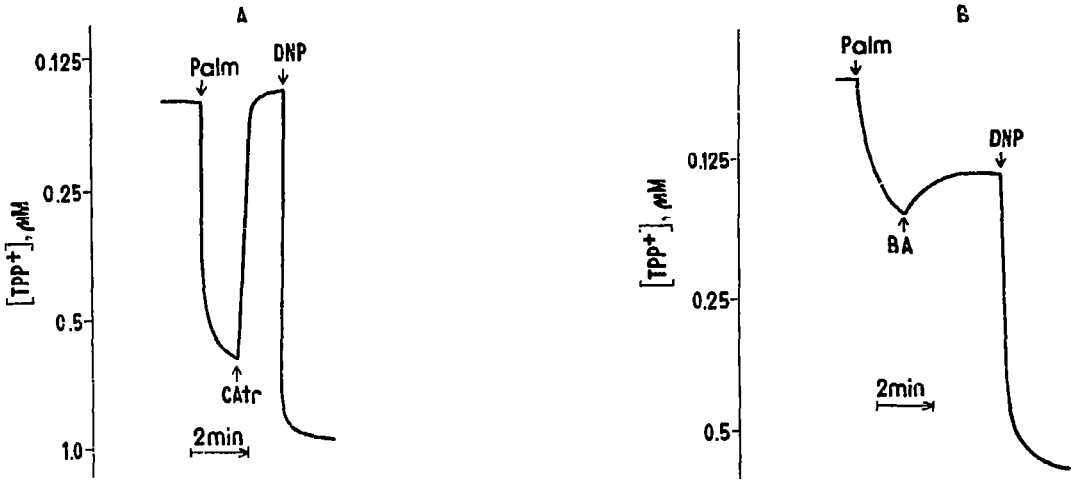

Fig. 2. Effects of CAtr and bongkrekic acid (BA) on palmitate-induced $\Delta\psi$ decrease in mitochondria. Incubation mixture: 0.25 M sucrose, 10 mM MOPS, 0.5 mM EGTA, 10 mM succinate, 1 μM rotenone, oligomycin (2 μg/ml), BSA (0.4 mg/ml), 1.5 μM TPP^+, pH 7.4, 26°C. Additions, mito, rat heart mitochondria (0.8 mg protein/ml); 1 μM CAtr; 20 μM DNP; 5 μM BA; 40 μM (A) and 20 μM (B) palmitic acid.

Just these relationships could be predicted assuming that it is the ATP/ADP antiporter that mediates the uncoupling effect of free fatty acids. It is known that the CAtr and bongkrekate binding sites of the antiporter are localized on outer and inner surfaces of the inner mitochondrial membrane, respectively, whereas sites for palmitoyl-CoA are on both membrane surfaces. It is also found that CAtr cannot penetrate through the membrane whereas bongkrekic acid can (for review see [5]).

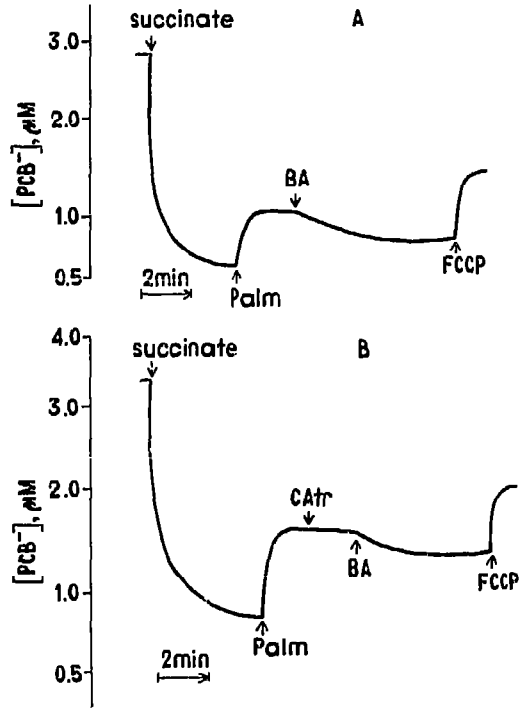

Fig. 3. Effects of bongkrekic acid, CAtr and FCCP on palmitate-induced $\Delta\psi$ decrease in submitochondrial particles. Incubation mixture: 0.25 M sucrose, 10 mM MOPS, 0.5 mM EGTA, and submitochondrial particles (0.45 mg protein/ml); 1 μM rotenone, oligomycin (4 μg/ml), 15 μM PCB^-, pH 7.3, 26°C. Additions, 5 mM succinate, 1 μM CAtr; BA, 8 μM bongkrekic acid; palm, 30 μM palmitic acid; 1 μM FCCP.

Thus comparison of heart mitochondria and particles gives one more piece of evidence that the ATP/ADP antiporter is somehow involved in the fatty acid uncoupling.

The results described for heart mitochondria confirmed our previous observations made with liver and skeletal muscle mitochondria [2–4], i.e. among the specific inhibitors of the antiporter, CAtr is the most potent agent for suppressing fatty acid-induced uncoupling, whereas ADP, Atr, palmitoyl CoA and bongkrekic acid are less effective. Atr added before CAtr prevents, at least for some time, complete inhibition of the palmitate-stimulated respiration by CAtr (see also [2]). It should be emphasized that the Atr concentration used was quite sufficient to completely inhibit oxidative phosphorylation. A probable explanation for this fact is that all the tested inhibitors combine with the interface-localized, substrate-specific antiporter gates rather than with the non-specific anion-translocating mechanism inlaid in the membrane core. Apparently, modification of gate decelerates the translocator mechanism and, as a result, inhibits the fatty acid anion-translocation rate. This deceleration may vary depending on the kind of inhibitor.

Acknowledgements: The authors are very grateful to Prof. A.D. Vinogradov for providing us with a preparation of submitochondrial particles and to Sigma-Tay for financial support.

REFERENCES

[1] Walter, A. and Gutknecht, J. (1984) J. Membr. Biol. 77, 255–264.

[2] Andreyev, A.Yu., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Tsofina, L.M., Volkov, N.I. and Vygodina, T.V. (1989) Eur. J. Biochem. 182, 585–592.
[3] Andreyev, A.Yu., Volkov, N.I., Mokhova, E.N. and Skulachev, V.P. (1987) Biol. Membrany 4, 474–478 (Russ.).
[4] Andreyev, A.Yu., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P. and Volkov, N.I. (1987) FEBS Lett. 226, 265–269.
[5] Skulachev, V.P. (1988) Membrane Bioenergetics, Springer, Berlin.
[6] Garlid, K.D. (1990) Biochim. Biophys. Acta 1018, 151–154.
[7] Jezek, P. and Garlid, K.D. (1990) J. Biol. Chem. 265, 19303–19311.
[8] Aquila, H., Link, T.A. and Klingenberg, M. (1985) EMBO J. 4, 2369–2376.
[9] Bouilland, F., Weissenbach, J. and Ricquier, D. (1986) J. Biol. Chem. 261, 1487–1490.
[10] Brustovetsky, N.N., Dedukhova, V.I., Yegorova, M.N., Mokhova, E.N. and Skulachev, V.P. (1990) FEBS Lett. 272, 187–189.
[11] Brustovetsky, N.N., Amerkhanov, Z.G., Yegorova, M.N., Mokhova, E.N. and Skulachev, V.P. (1990) FEBS Lett. 272, 190–192.
[12] Brustovetsky, N.N., Amerkhanov, Z.G., Yegorova, M.N., Mokhova, E.N. and Skulachev, V.P. (1991) Biokhimiya 56, 947–953 (Russ.).
[13] Schonfeld, P. (1990) FEBS Lett. 264, 246–248.
[14] Kotlar, A.B. and Vinogradov, A.D. (1990) Biochim. Biophys. Acta 1019, 151–158.
[15] Grinius, L.L., Jasaitis, A.A., Kadziauskas, J.P., Liberman, E.A., Skulachev, V.P., Topali, V.P. and Vladimirova, M.A. (1970) Biochim. Biophys. Acta 216, 1–12.
[16] Kamo, N., Muratsuga, M., Hongoh, R. and Kobatake, Y. (1979) J. Membr. Biol. 49, 105–121.